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APPLICATION NUMBER: *60/507,677*
FILING DATE: *September 30, 2003*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
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7858 U.S.PTO 60/507677 093003

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EU 980965496 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
ANTONIO	GIORDANO	RADNOR, PA

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

GENE MODULATION BY RB2/P130 EXPRESSION

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number:

OR

☐ Firm or Individual Name	REED SMITH				
Address	2500 One Liberty Place				
Address	1650 Market Street				
City	Philadelphia, PA	State	PA	Zip	19103-7301
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 11 (including 5 figures)
☐ CD(s), Number _____
☐ Drawing(s) *Number of Sheets* _____
☐ Other (specify) _____
☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees.
☒ The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 18-0586
☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☒ Yes, the name of the U.S. Government agency and the Government contract number are: NIH R01 CA60999-08

Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME NANDA P.B.A. KUMAR

TELEPHONE (215) 241-7991

Date 9/30/2003

REGISTRATION NO. 44,853 *(if appropriate)*

Docket Number: 03-4071-US PR

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PTO/SB/17 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($)

Complete If Known	
Application Number	TBD
Filing Date	SEPTEMBER 30, 2003
First Named Inventor	GIORDANO
Examiner Name	
Art Unit	
Attorney Docket No.	03-40171-USPR

METHOD OF PAYMENT *(check all that apply)*

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 80.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

(Complete *(if applicable)*)

SUBMITTED BY					
Name (Print/Type)	NANDA P.B.A. KUMAR	Registration No. (Attorney/Agent)	44,853	Telephone	(215) 241-7991
Signature				Date	September 30, 2003

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

ReedSmith

Nanda P.B.A. Kumar
Direct Phone: 215.241.7991
Email: nkumar@reedsmith.com

2500 One Liberty Place
1650 Market Street
Philadelphia, PA 19103-7301
215.851.8100
Fax 215.851.1420

EXPRESS MAIL CERTIFICATE (37 CFR 1.10)

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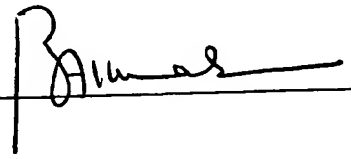
Date of Deposit September 30, 2003

I hereby certify that this paper, and the papers and/or fees referred to herein as transmitted, submitted or enclosed, are being deposited with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on the date indicated above and is addressed to MS Provisional Patent Application, Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1420.

Name: Nanda P.B.A. Kumar

Signature 

September 30, 2003

MS Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RE: New Provisional Patent Application
Applicant: Thanoo
Filing Date: herewith
For: GENE MODULATION BY RB2/p130 EXPRESSION
Docket No. 03-40171-USPR (869233.20001)

Dear Sir:

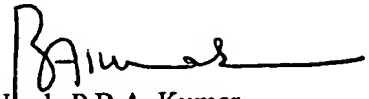
Enclosed are the following for filing in connection with the above-referenced application:

1. Provisional Application For Patent Cover Sheet;
2. Fee Transmittal for FY 2003;
3. A check in the amount of $80.00 to cover the filing fee for a provisional application;
4. Application consisting of 74 pages of specification and 19 sheets of drawings; and
5. A self-addressed stamped postcard, return of which is requested to acknowledge receipt of the enclosed documents.

ReedSmith

The Commissioner is hereby authorized to charge any fees due in connection with this filing to Deposit Account No. 18-0586.

Respectfully submitted,



Nanda P.B.A. Kumar
Registration No. 44,853

NPK
Enclosures

GENE MODULATION BY RB2/P130 EXPRESSION

The retinoblastoma gene family consisting of *RB1/p105*, *p107*, and *RB2/p130* cooperate to regulate cell-cycle progression through the G1 phase of the cell cycle. Previous data demonstrated an independent role for the reduction or loss of pRb2/p130 expression in the formation and/or progression of lung carcinoma. Rb2/p130 is mutated in a human cell line of lung small cell carcinoma as well as in primary lung tumors. To identify potential pRb2/p130 target genes in an unbiased manner, we have utilized an adenovirus-mediated expression system of pRb2/p130 in a non-small lung cancer cell line to identify specific genes that are regulated by pRb2/p130. Using oligonucleotide arrays, a number of Rb2/p130 downregulated genes were identified and their regulation was confirmed by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) and Western blot analysis. As a result, 40 genes showed greater than 2.0-fold modification in their expression level after the *RB2/p130* viral transduction. In conclusion, coupling adenoviral overexpression with microarray and semiquantitative RT–PCR analyses proved to be a versatile strategy for identifying pRb2/p130 target genes and for better understanding the expression profiles of these genes. Our results may also contribute to identifying novel therapeutic biomarkers in lung carcinoma.
Oncogene (2003) 0, 000–000. doi:10.1038/sj.onc.1206866

Keywords: lung cancer; Rb2/p130; microarray analysis

Introduction

Lung cancer is the leading cause of cancer death worldwide and one of the most common malignancies diagnosed in the United States. The American Cancer Society estimated that 169 500 new cases of lung cancer would be diagnosed in 2001 and that 157 400 people would die of the disease that year. Data from the American Cancer Society for the year 2002 indicate that 169 400 new cases of lung cancer are estimated to occur and 154 900 patients are expected to die because of lung cancer (Greenlee *et al.*, 2001). Additionally, lung cancer is usually diagnosed at an incurable stage.

The high mortality rate for lung cancer probably results from the absence of effective therapies as well as standard diagnostic procedures of early tumoral stages compared with colon, breast, and prostate cancers (Wiest *et al.*, 1997). The majority of bronchogenic carcinomas can be classified into four histological types: small cell lung carcinomas, adenocarcinomas, squamous cell lung carcinomas, and large cell carcinomas. Small cell lung carcinomas are a separate entity, whereas the behavior of the other three histological subtypes is similar, for this reason these are grouped within the non-small cell lung cancer (NSCLC) type. NSCLC accounts for nearly 80% of lung malignant tumors and it is associated with a poor prognosis.

Lung cancer is the result of molecular changes in the cell, resulting in the deregulation of pathways that control normal cellular growth, differentiation, and apoptosis. In this scenario, proto-oncogenes and tumor suppressor genes are found to be mutated or have abnormal expression patterns in this disease. Although much is known about the natural history, predisposing factors, and outcome of NSCLC, the understanding of this disease is still unclear. Even though many molecular changes associated with NSCLC have been reported (Hibi *et al.*, 1998; Forgacs *et al.*, 2001), a fully understood mechanism associated with this type of cancer has not been described yet.

The retinoblastoma gene family consisting of *RB1*, *p107*, and *RB2/p130* cooperate to regulate cell-cycle progression through the G1 phase of the cell cycle. Products of *RB1*, *p107*, and *RB2/p130* are characterized by a peculiar steric conformation, the 'pocket region', which is responsible for most of the functional interactions that characterize the activity of these proteins in cell-cycle homeostasis (Paggi *et al.*, 1996). Rb family members are nuclear proteins that are regulated in a cell-cycle-dependent manner by phosphorylation, exhibit growth suppressive properties in a cell-type-dependent

manner, are implicated in various forms of differentiation, and are critical targets for inactivation by transforming oncoproteins of DNA tumor viruses.

During the past 6 years, the involvement of pRb2/p130 in lung cancer has been studied. In fact, immunohistochemical analysis of the expression patterns of the Rb family members (pRb/p105, p107, and pRb2/p130) in 235 specimens of lung cancer (Baldi *et al.*, 1996) and the expression pattern of pRb2/p130 in 158 specimens of human lung cancer showed an inverse correlation between the histological grading of the tumors, the development of metastasis, and the level of expression of pRb2/p130 (Baldi *et al.*, 1997). A statistically significant inverse relationship between the histological grading and the expression of pRb/p105, p107, and pRb2/p130 was found in fine needle aspiration biopsies of squamous cell carcinoma patients (Minimo *et al.*, 1999).

Point mutation in the *RB2/p130* gene determining protein loss in a cell line of human small cell lung carcinoma (Helin *et al.*, 1997) and identification of point mutations in primary lung cancer (Claudio *et al.*, 2000) were found, indicating that pRb2/p130 may play a role in the pathogenesis and progression of certain lung cancers.

We have utilized an adenovirus-mediated expression of *RB2/p130* in the H23 NSCLC cell line to identify specific genes that are regulated by pRb2/p130. Using oligonucleotide arrays, a number of downregulated genes by pRb2/p130 were identified and their modulation was confirmed by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) and Western blot analysis.

Materials and methods

Cell lines

The human lung adenocarcinoma cell line H23 has been described previously (Claudio *et al.*, 2000). The packaging cell line 293 (primary embryonal human kidney cells) transformed by sheared human adenovirus type 5 has also been previously described (Claudio *et al.*, 1999). H23 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine. 293 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine.

Adenoviruses

Adenoviruses were generated by subcloning the full-length ORF of the *RB2/p130* gene into the pAd.CMV-Link1 vector to form the Ad.CMV-*RB2/p130* virus, as described previously (Davis *et al.*, 1998; Claudio *et al.*, 1999). The pAd.CMV-Link1 vector alone (to produce the Ad-CMV virus) was used as a negative control to assay the effects of viral infection alone without delivering a transgene. Adenoviruses were expanded, purified, and tittered as previously described (Claudio *et al.*, 1999).

Flow cytometry analysis

Flow cytometry analysis (FACS) of exponentially growing H23 cells or H23 cells transduced with Ad-CMV or Ad-CMV-Rb2/p130 were carried out as previously described (Claudio *et al.*, 1996). Briefly, 5×10^5 cells were seeded and 24 h after the cells were transduced with 50 multiplicity of infection (MOI) of adenoviruses. At 48 h after transduction, cells were collected and analysed using a Coulter Flow cytometer.

Microarray analysis

Before submission of RNA samples for analysis, protein extracts prepared from replicate plates of the corresponding cell culture were analysed for expected enhanced expression of pRb2/p130 using Western blots. Oligonucleotide-based microarrays were purchased from Mergen (Mergen Ltd, San Leandro, CA, USA). The ExpressChip H05000 DNA microarray system was used for this study. This array contains more than 3200 genes that are involved in a variety of different processes. DNase-treated total RNA (20 μg) from H23 (parental cells), H23 cells transduced with Ad-CMV, or Ad-CMV-RB2/p130 cell lines 48 h after transduction were extracted using TRIzol (Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's protocol. RNA integrity was verified for lack of degradation by formaldehyde gel electrophoresis. The biotin-labeled cRNA probes preparation, hybridization, and array scanning were performed using Mergen Labeling/Hybridization/Detection Service. Data acquisition and data analysis were performed using Imagene software (Biodiscovery Inc., Marina del Rey, CA, USA) and Mergen's *Express*Data™ software (Mergen Ltd, San Leandro, CA, USA). Briefly, data were processed for local background correction and normalization. The raw spot for each gene was calculated as the mean signal of the spot values minus that of the local background. The I_{max} value was set to 65 000, after local background removal. A normalization coefficient (N) was applied to either the control population or to sample spot raw values to compensate for slide-to-slide and probe-to-probe variations. The normalization coefficient was applied only if $(\text{Raw\_spot}/N) < I_{max}$, otherwise values were set to I_{max}. Normalized values $\leqslant 0$ were excluded from the analysis. Genes regulated by adenovirus transduction (Ad-CMV) with respect to the parental cell line were removed from the analysis. Spots with mean intensities >45 000 were excluded for the ratio analysis. The expression ratios calculated with corrected values less than mean of the local background on both channels were not used. Expression ratios of the analysed genes were calculated comparing genes' expression values of H23 cells transduced with RB2/p130 with those of parental H23 or H23 cells transduced with Ad-CMV. A twofold or higher levels of target genes' expression ratio was considered significant, in accordance with most of the literature.

Northern blot analysis

H23 cells were grown to 60% confluency, then infected with 50 MOI of adenoviruses carrying the *RB2/p130* gene or with the control Ad-CMV. After 14 h, the medium was changed, and cells were harvested at 48 h from the transduction. DNAse-treated total RNA from H23, H23-Ad-CMV, and H23-Ad-CMV-Rb2/p130 transduced cells were extracted using TRIzol (Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's protocol. Northern blot analysis was performed as previously described (Claudio *et al.*, 1994).

Semiquantitative RT–PCR

RT–PCR was used to analyse target gene expression in the present study. A 2 μg aliquot of DNAse-treated total RNA from each sample was reverse transcribed for single-stranded

cDNAs using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The same cDNA product obtained from each sample was used for subsequent PCR amplification with the primer sets prepared for the target gene and *β-actin* (Act-β)/*HPRT* housekeeping genes. The amplification of the *β-actin* and *HPRT* genes were used as double internal control. The ratio between the samples and each housekeeping gene was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Primer sequences were designed using the software Primer 3 (developed by Steve Rozen, Helen J Skaletsky) available on-line at http://www-genome.wi.mit.edu. Primer sequences can be provided upon request. PCR reaction conditions were individually optimized for each gene product studied and the number of PCR cycles was set up to be within the linear range of product amplification.

In each experiment, possible DNA contamination was determined by a control reaction in which reverse transcriptase was omitted from the reaction mixture. PCR products were loaded onto ethidium bromide stained 1.5% agarose gels. Densitometric analyses of the PCR products were performed using an Alpha Imager system (Alpha Innotech Corporation, San Leandro, CA, USA) and the ImageJ v1.29 software (developed by Wayne Rasband) available on-line at http://rsb.info.nih.gov/ij/. All PCR products were purified using QIAquick PCR purification kit (Qiagen, Santa Clarita, CA, USA) and their identities verified by automated DNA forward and reverse sequencing using a dideoxy terminator reaction chemistry for sequence analysis on the Applied biosystem Model 373A DNA sequencer.

Western blot analysis and antibodies

Western blot analysis of exponentially growing H23 cells or of H23 cells transduced with Ad-CMV or Ad-CMV-Rb2/p130 were carried out as previously described (Claudio *et al*., 1999). Extracts were normalized for protein content by Bradford analysis (Bio-Rad Laboratories, Inc., Melville, NY, USA) and Coommasie blue gel staining.

Primary anti-B-MYB, E2F-1, KPNA2, MKK3, NIK, PCNA, PLK, RAF1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-MAGE-A (Upstate, Lake Placid, NY, USA), and anti-HSP70 (Oncogene Science, Cambridge, MA, USA) were used following the manufacturer's instructions.

Results

Effects of RB2/p130 adenoviral transduction on the H23, lung adenocarcinoma cell line

H23 cells were plated at a density of 5×10^5 in four 10-cm tissue culture dishes. Cells were transduced with 50 MOI of the control Ad-CMV or Ad-CMV-*RB2/p130* and harvested after 48 h. Two tissue culture dishes were used to extract mRNA. One tissue culture dish was used to extract the proteins and one for FACS analysis.

Northern blot analysis of samples transduced with *RB2/p130* showed an increased expression of the *RB2/p130* transcript more than 20-fold with respect to the control (Figure 1a). Western blot analysis showed more than 100-fold enhanced expression of pRb2/p130 in the Ad-CMV-*RB2/p130* transduced cells (Figure 1b).

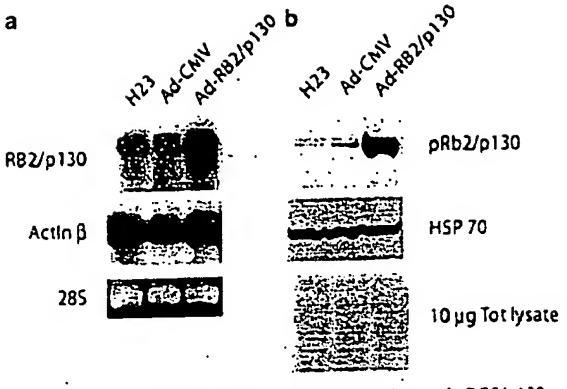


Figure 1 Adenovirus-mediated overexpression of RB2/p130. Northern blot and Western blot analyses (a, b) of RB2/p130 in H23, H23-Ad-CMV, and H23 Ad-CMV-RB2/p130 NSCLC cell line

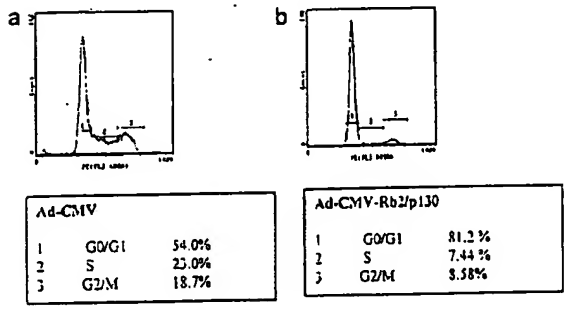


Figure 2 Effects of pRb2/p130-enhanced expression in H23 cells. FACS analysis of H23-Ad-CMV (a) and H23-Ad-CMV-Rb2/p130 (b) infected cells. Rb2/p130 overexpression resulted in 81.2% of the cells being accumulated in the G0/G1 phase of the cell cycle when compared to the empty adenovirus (54%). The analysis was performed in triplicates with comparable results

To confirm the effects of pRb2/p130-enhanced expression in H23 cells, we performed FACS analysis. Figure 2 shows that adenoviral Rb2/p130 transduction resulted in 81.2% of the cells accumulated in the G0/G1 phase of the cell cycle when compared to the control (54%).

Oligonucleotide microarray assay following enhanced expression of pRb2/p130 in a human lung adenocarcinoma cell line

H23 cells were transduced with Ad-CMV or Ad-CMV-*RB2/p130*. After 48 h, 20 µg of DNA-free total RNA from H23, H23-Ad-CMV, or H23-Ad-CMV-*RB2/p130* cells was reverse transcribed and the double-strand cDNA was used as a template to generate Cy3-labeled cRNA probes and then hybridized to the Mergen H05000 oligonucleotide-based microarray containing more than 3200 genes that are involved in a variety of different processes. Analysis was performed by Mergen Ltd (San Leandro, CA, USA). Microarray experiments were performed comparing H23 *vs* H23-Ad-CMV, H23-Ad-CMV *vs* H23-Ad-CMV-*RB2/p130*, and H23 *vs*

H23-Ad-CMV-*RB2/p130* cells. Duplicate experiments were carried out on a single total RNA preparation from the cells.

In this study, 40 genes were downregulated more than 2.0-fold (Table 1). Figure 3 shows the plots of the differential expression of 3263 genes in H23-Ad-CMV *vs* H23-Ad-CMV-*RB2/p130* cells and H23 *vs* H23-Ad-CMV-*RB2/p130* cells. Overall, the expression of the majority of the spotted genes was not altered by *RB2/p130*. Modulated genes were classified in Table 2 on the basis of a well-documented and established biological or pathological function of the encoded protein. The genes downregulated by pRb2/p130-enhanced expression belong mainly to the following categories: cell division; signaling and communication; cell structure and motility; gene expression; metabolism; and disease.

Validation of the oligonucleotide microarray assay using semiquantitative RT–PCR and Western blot analysis

To determine the gene expression level of specific pRb2/p130 target genes, semiquantitative RT–PCR analysis was used. A panel of 11 genes, randomly selected among the 40 identified by microarray analysis, was analysed. We confirmed this by RT–PCR downregulation of *B-MYB*, *Cyc B2*, *Cyc D1*, *GRPR*, *KPNA2*, *MKK3*, *NIK*, *PCNA*, *PIM*, *PLK*, and *RAF-1* (Figure 4). Genes highly downregulated (range between 6- and 17-fold) in microarray analysis such as *PCNA*, *MKK3*, *B-MYB*, and *NIK* showed a comparative downregulation in semiquantitative RT–PCR analysis between 7- and 3.5-fold. Genes still downregulated in microarray analysis, but at a lower extent such as *RAF-1*, *PIM1*, *CycD1*, *GRPR*, *KPNA2*, and *CycB2*, showed a comparable

Table 1 Downregulated genes by Rb2/p130 adenovirus-enhanced expression

	GenBankTM ID	*Gene description*	*Gene symbol*	*Ratio 1*	*Ratio 2*	*Average*
1	M15796	Proliferating cell nuclear antigen	PCNA	16.4	17.5	16.9
2	L36719	Mitogen-activated protein kinase kinase 3	MKK3	12.4	19.2	15.8
3	X13293	V-myb avian myeloblastosis viral oncogene homolog-like 2	B-MYB	8.2	13	10.6
4	Z36714	Cyclin F	CCNF	6.6	8.7	7.6
5	AF053306	Budding uninhibited by benzimidazoles 1 (yeast homolog), β	BUB1B	8.9	6	7.4
6	L19559	Polo (Drosophia)-like kinase	PLK	6.5	7.5	7
7	Y10256	Mitogen-activated protein kinase kinase kinase kinase 4	NIK	5.6	7.6	6.6
8	D14678	Kinesin-like 2	KNSL2	5	6.7	5.8
9	U33849	Proprotein convertase subtilisin/kexin type 7	PCSK7	4.4	6.5	5.4
10	AF002822	Cyclin B2	CCNB2	5.3	4.2	4.7
11	L16862	G protein-coupled receptor kinase 6	GPRK6	3.5	5.7	4.6
12	L20010	Host cell factor C1	HCFC1	3.2	5.9	4.5
13	AB002359	FGAR amidotransferase	PFAS	3.7	5	4.3
14	X63692	DNA (cytosine-5-)-methyltransferase 1	DNMT1	3.5	4.9	4.2
15	U09559	Karyopherin α 2	KPNA2	3.5	4.1	3.8
16	AF011468	Serine/threonine kinase 15	STK15	3.7	4	3.8
17	U21847	TGFB-inducible early growth response	TIEG	3.3	4.3	3.8
18	F046078	Budding uninhibited by benzimidazoles 1 (yeast homolog)	BUB1	4.3	3.1	3.7
19	M25269	ELK1, member of ETS oncogene family	ELK1	3.1	3.1	3.1
20	D78335	Uridine monophosphate kinase	UMPK	3.2	3.1	3.1
21	X51804	Putative receptor protein	PMI	2.4	3.7	3
22	AB018330	Calcium/calmodulin-dependent protein kinase kinase 2, β	CAMKK2	2.1	3.7	2.9
23	L33801	Glycogen synthase kinase 3 β	GSK3B	3.2	2.6	2.9
24	AF001903	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	HADHSC	3.2	2.6	2.9
25	M81735	Polymerase (DNA directed), δ 1, catalytic subunit	POLD1	2.5	3.3	2.9
26	M32110	Nucleolar protein 1	NOL1	2.7	3	2.8
27	X97630	ELKL motif kinase	EMK1	2.4	3.3	2.8
28	M73481	Gastrin-releasing peptide receptor	GRP-R	2.7	2.7	2.7
29	AF035586	X-ray repair complementing defective repair in Chinese hamster cells 3	XRCC3	2.5	3	2.7
30	D10704	Choline kinase	CHK	3	2.3	2.6
31	U10339	Melanoma antigen, family A,3/6	MAGEA3/6	2.1	3	2.5
32	Y13936	Protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	PPM1G	2.3	2.8	2.5
33	AB000509	TNF receptor-associated factor 5	TRAF5	2.2	2.7	2.4
34	AJ005016	ATP-binding cassette, sub-family F (GCN20), member 2	ABCF2	2.4	2.5	2.4
35	U6382	TEA domain family member 4	TEAD4	2.5	2.2	2.3
36	M54915	Pim-1 oncogene	PIM1	2.2	2.3	2.2
37	X59798	Cyclin D1	CCND1	2.1	2.1	2.1
38	M63256	Cerebellar degeneration-related protein	CDR2	2.2	2.1	2.1
39	D26599	Proteasome subunit, β type, 2	PSMB2	2.2	2.1	2.1
40	X03484	V-raf-1 murine leukemia viral oncogene homolog 1	RAF1	2.3	2	2.1

Genes that are downregulated more than 2.0-fold in response to the enhanced expression of RB2/p130 by microarray analysis are listed. Genes were identified as unique as mentioned in the GenBank™ and are sorted in descending order. Ratio 1 indicates the fold of repression for each gene as determined by microarray analysis of H23-Ad-CMV *vs* H23-Ad-CMV-RB2/p130. Ratio 2 indicates the fold of repression for each gene as determined by microarray analysis of H23 *vs* H23-Ad-CMV-RB2/p130

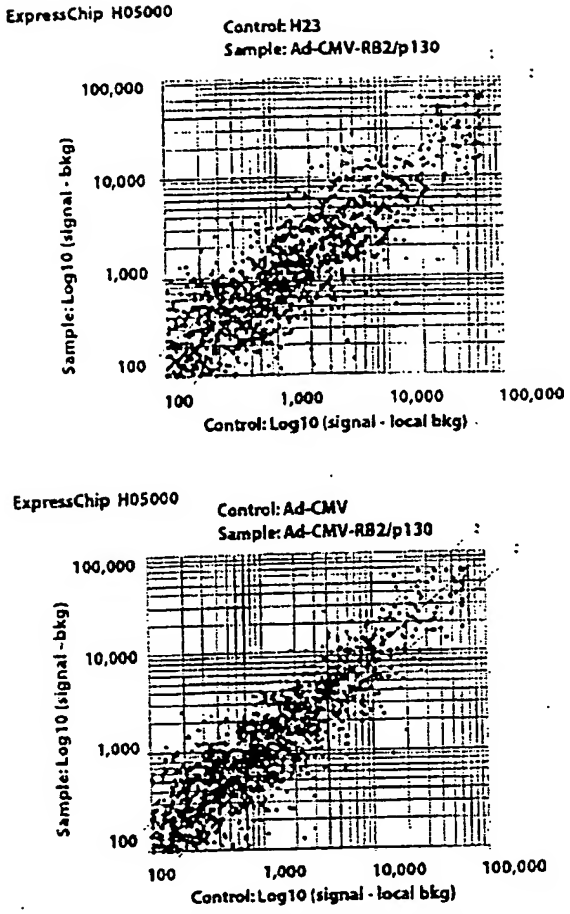


Figure 3 Global comparison of gene expression in H23 *vs* H23-Ad-CMV and H23-Ad-CMV *vs* H23-Ad-CMV-RB2 cells. Each *dot* corresponds to the Cy3 fluorescent intensity of one single element on the oligonucleotide microarray. A twofold change in expression is indicated with parallel lines marked as 2

downregulation in semiquantitative RT–PCR analysis between 3.4- and 2.0-fold. *PLK*, which showed a high downregulation ratio in microarray analysis, failed to be validated by semiquantitative RT–PCR. In fact, *PLK* showed almost a twofold difference expression level by RT–PCR.

Of the 11 transcriptionally downregulated genes that were studied by RT–PCR analysis, only seven genes (*B-MYB*, *KPNA2*, *MKK3*, *NIK*, *PLK*, and *RAF-1*) were found expressed by Western blot analysis at a lower level upon enhanced pRb2/p130 expression with a ratio between 1.9- and 3.0-fold (Figure 5). As the *MAGE* gene family is composed of 23 related genes divided into four clusters and the *MAGE-A* subfamily comprises 12 genes highly identical in their coding sequence, we were not able to perform RT–PCR on this gene family, but we could confirm by Western blot analysis the contingent downregulation of *MAGE-A-3/6* to enhanced pRb2/p130 expression. Surprisingly, *PCNA* that was highly downregulated in the microarray analysis, also appearing modulated in RT–PCR, showed no protein expression

Table 2 Classification of RB2/p130-repressed genes by category

Category	*Genes*
ATPase/GTPase/ATP binding/GTP binding	ABCF2 KNSL2 CAMKK2
Calcium/potassium/sodium/iron binding protein	
Cell cycle/cyclins	BUB1 BUB1B CCNB1 CCNB2 CCND1 CCNF B-MYB NOL1 PCNA PLK PPM1G
Cell surface/antigen	ABCF2 CDR2 GPRK6 GRP-R KNSL2 MAGE-A 3/6 PCNA PMI
Chromosome/chromatin/histone	DNMT1 PLK XRCC3
Cytokines and growth factors	GRP-R PCSK7 TIEG TRAF5
Cytoskeleton/microtubules/microfilaments/motility	CAMKK2 EMK1 KNSL2
Differentiation/development	BUB1 GSK3B B-MYB NOL1 PIM1 PLK RAF1 TEAD4 TIEG
Diseases	B-MYB CCND1 CDR2 ELK1 MAGE-A 3/6 NOL1 PCSK7 PIM1 RAF1 STK15
DNA binding/damage/recombination	DNMT1 POLD1 XRCC3
G protein/regulators of G protein signaling	GPRK6 GRP-R PMI
Hydrolase/hydrolysis/hydrolyses	ABCF2 PCSK7 PPM1G

Table 2 Continued

Category	*Genes*
Kinases	BUB1
	BUB1B
	CAMKK2
	CHK
	ELK1
	EMK1
	GPRK6
	GSK3B
	MKK3
	NIK
	PLK
	RAF1
	STK15
	UMPK
Lipoproteins/lipids	CHK
Membrane trafficking	DNMT1
	KPNA2
Mitochondrial proteins	ABCF2
	HADHSC
Nuclear receptors/receptors	BUB1
	DNMT1
	ELK1
	GPRK6
	GRP-R
	NOL1
	PCNA
	PMI
	POLD1
	PPM1G
	TIEG
	TRAF5
Oncogenes	B-MYB
	ELK1
	PIM1
	RAF1
Phosphatase/proteases/peptidase	PCSK7
	PPM1G
	PSMB2
Signal transduction	CAMKK2
	GPRK6
	GRP-R
	MKK3
	NIK
	PMI
	TRAF5
Synthetase/synthase	GSK3B
	PFAS
Transcription/transcription factor	B-MYB
	CDR2
	ELK1
	HCFC1
	TEAD4
	TIEG
Transporters	ABCF2
Transferases	DNMT1
	PFAS

Classifies the analysed genes on the basis of established biological or pathological functions of the encoded proteins. Genes that are listed in one category are indicated in bold

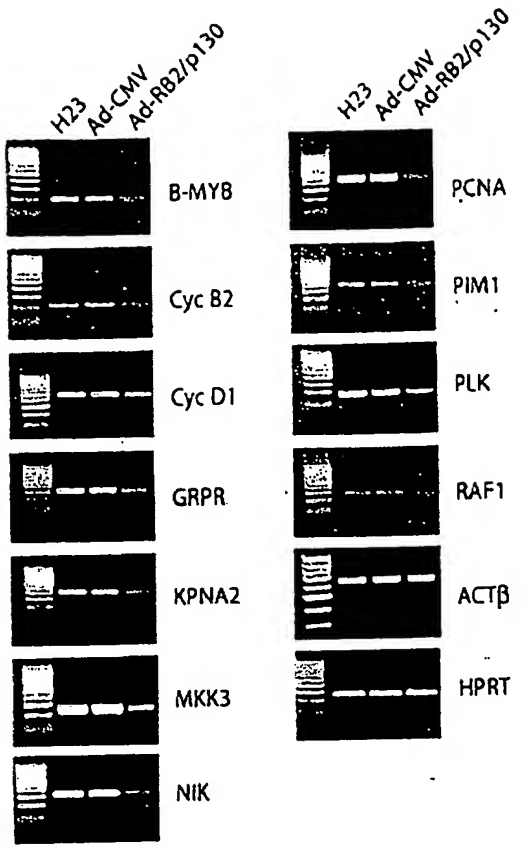


Figure 4 Validation of oligonucleotide microarray results of 11 selected genes by semiquantitative RT-PCR. RT-PCRs were performed using DNAse-treated total RNA of H23, H23-Ad-CMV, and H23-Ad-CMV-Rb2/p130 NSCLC cell line. Amplified fragments of B-MYB (194 bp), Cyc B2 (217 bp), Cyc D1 (463 bp), GRPR (377 bp), KPNA2 (304 bp), MKK3 (219 bp), NIK (317 bp), PCNA (420 bp), PIM1 (324 bp), PLK (154 bp), and RAF1 (280 bp) genes are indicated. ACT-β (626 bp) and HPRT (349 bp) genes were used as internal controls and were amplified from the same samples

changes upon enhanced pRb2/p130 expression. However, it has been shown that PCNA has a relatively long half-life that can extend beyond the S phase into the M phase and beyond into the G0 phase of cells in rapidly proliferating tumors.

Discussion

During the last 6 years, different studies demonstrated the involvement of *RB2/p130* in lung cancer. We have shown an inverse correlation between histological grading of lung cancer and the expression pattern of the tumor suppressor gene pRb2/p130 (Baldi *et al.*, 1996, 1997; Minimo *et al.*, 1999). Nevertheless, the molecular mechanisms by which Rb2/p130 participate in lung cancer are not yet clear.

In the recent past, our group demonstrated that virally enhanced expression of pRb2/p130 caused about

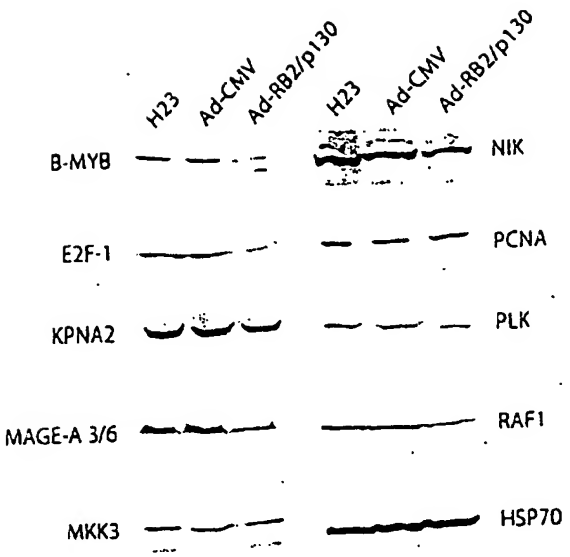


Figure 5 Validation of oligonucleotide microarray data by Western blot analysis. In all, 100 μg of protein extracts from H23, H23-Ad-CMV, and H23-Ad-CMV-Rb2/p130 cells were loaded onto SDS–PAGE gels and immunoblotted with antibodies anti B-MYB, E2F-1, MAGEA-3/6, MKK3, NIK, PCNA, PLK, and RAF1. Anti-HSP-70 was used as an internal control. The analysis was performed in duplicates with comparable results

a 3.2-fold or 69% tumor mass reduction in *nude* mice. These data strongly suggest the gene therapeutical potential of *RB2/p130* in lung cancer. In this view, the identification of genes modulated by enhanced pRb2/p130 expression could serve as a valuable tool in identifying new targets for gene therapy in lung cancer as well as to understand the molecular mechanisms behind this approach better. For these reasons, we decided to analyse the NSCLC H23 cells transduced with pRb2/p130.

In our study, we induced an overexpression on the *RB2/p130* gene in H23 cells (human lung adenocarcinoma) using an adenoviral system and performed an oligonucleotide microarray analysis to measure and evaluate the gene's expression profile. We identified 40 genes that were downregulated more than 2.0-fold by pRb2/p130 adenovirus-mediated overexpression. Regulated genes were summarized in 24 categories as shown in Table 2. We applied a cutoff ratio of 2.0 that has been commonly used in many microarray data analyses previously developed. We confirmed the modulation of 11 selected genes by semiquantitative RT–PCR and of six by Western blot analysis (Figures 4 and 5).

B-MYB in our microarray analysis showed an almost 10-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction. Downregulation of *B-MYB* was confirmed by RT–PCR and Western blot analysis. *B-MYB* is a member of a family of transcription factors, which include *c-MYB* and *A-MYB*, that interact with the promoters of different genes involved in differentiation as well as cell cycle (Lyon *et al.*, 1994). It has been demonstrated that B-Myb can activate promoters, both via DNA binding-dependent and -independent mechanisms, following phosphorylation mediated by cyclin A/E-cdk2 kinase (Lane *et al.*, 1997; Sala *et al.*, 1997). *B-MYB* is a positive regulator of cell proliferation and its expression is required for the growth and survival of cells of different origins. *B-MYB* expression is stringently coupled to the proliferative state of the cell, and its overexpression promotes cell proliferation (Sala *et al.*, 1996). It has been reported that pRb2/p130 increases during neuroblastoma differentiation with a parallel decrease of B-Myb (Raschella *et al.*, 1997). Additionally, there is a large body of evidence that the negative regulation of *B-MYB* takes place, at least in part, at a transcriptional level (Raschella *et al.*, 1996), through an E2F site in the promoter region of *B-MYB* when RB family proteins form complexes with E2F factors bound to DNA (Zwicker *et al.*, 1996).

Recently, it was also demonstrated that B-Myb is abundantly expressed in primary lung cancers, suggesting that its overexpression may contribute to lung tumorigenesis (Hibi *et al.*, 1998). Our microarray data corroborating those of RT–PCR and Western blotting are in accordance with previously published studies.

Cyclin B2 (*Cyc B2*) in our microarray analysis showed a 4.7-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction. Downregulation of *Cyc B2* was confirmed by RT–PCR. Cyc B2 is a member of the cyclin family and a component of the B type. Recent data suggest that overexpression of human cyc B2, not due to mere gene amplification, could contribute through an alteration of the spindle checkpoint and chromosomal segregation, and to chromosomal instability of cancer cells, which is a phenotype persistently present in human cancers including lung cancer (Masuda and Takahashi, 2002).

Cyclin D1 (*Cyc D1*) showed a 2.1-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *Cyc D1* was confirmed by RT–PCR. Cyc D1 is a cell-cycle regulator essential for G1 phase progression and a candidate proto-oncogene implicated in the pathogenesis of several human tumor types. The *Cyc D1* gene maps to one of the most frequently amplified chromosomal regions (11q13) in human carcinomas (Motokura and Arnold, 1993) and it is frequently amplified in breast and lung cancer (Keyomarsi and Pardee, 1993). Additionally, *Cyc D1* is frequently overexpressed in the absence of genetic amplification in breast cancers (Gillett *et al.*, 1994) and its overexpression is associated with advanced local invasion and the presence of lymph node metastases in head and neck carcinomas (Muller *et al.*, 1997). *Cyc D1* is amplified and overexpressed in NSCLC (Reissmann *et al.*, 1999) and its expression has been shown to be a negative prognostic marker in lung cancer (Caputi *et al.*, 1999), confirming the hypothesis of Schauer *et al.* (1994). In fact, it has been suggested that lung tumors may evade cell-cycle control through abnormal expression of cyc D1. Additionally, cyc D1 overexpression and lack of expression of retinoblastoma protein is frequently seen in lung cancer.

In our microarray, *E2F-1* was one of the genes that, unfortunately, resulted in not being statistically significant. However, because recently it was shown that *E2F-1* can act either as an oncogene or as a tumor suppressor gene and that E2F-1 overexpression may contribute to the development of NSCLCs by promoting proliferation (Gorgoulis *et al.*, 2002), we performed a promoter analysis of all the 11 selected genes using the TESS software searching for E2F-1 and B-Myb DNA-binding regulatory regions. We found that the *GRPR*, *MAGE-A 3/6*, *MKK3*, *RAF1*, *B-MYB*, *PLK*, *PCNA*, *PIM1*, *Cyc B2* and *Cyc D1* genes contain E2F-1 and/or B-Myb DNA-binding sites in their promoter sequences. All these data strongly validate and explain the downregulation of *GRPR*, *MAGE-A 3/6*, *MKK3*, *RAF1*, *B-MYB*, *PLK*, *PCNA*, *PIM1*, *Cyc B2*, and *Cyc D1* in our system. In fact, the enhanced expression of pRb2/p130 downregulates *E2F-1* and *B-MYB* expression (Figure 5). Interestingly, the promoter sequence of the *NIK* gene that does not contain E2F-1 and/or B-Myb DNA-binding sites is also downregulated by enhanced pRb2/p130. No data are available for the human promoter sequence of the *KPNA2* gene, instead.

The gastrin-releasing factor receptor gene (*GRP-R*) showed a 2.7-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *GRP-R* was confirmed by RT–PCR. *GRP-R* is a member of the G protein-coupled receptor family and mediates important physiological actions of its specific ligand, the gastrointestinal hormone GRP. Many studies have found that GRP increases tumor cellular proliferation, leading to the hypothesis that this peptide hormone is a mitogen important for the growth of various cancers (Rozengurt, 1988). *GRP* has been implicated in the development of lung epithelium (Hoyt *et al.*, 1993) and it is thought to be a growth factor involved in human lung carcinoma (Fathi *et al.*, 1996). GRP-R has mitogenic activity and it is produced in an autocrine fashion in small and non-small lung carcinoma (Siegfried *et al.*, 1999). Therefore, the preliminary data that pRb2/p130 downregulates the transcription of this mitogen in lung cancer is an important hint that many and different pathways may be regulated by this member of the retinoblastoma family.

The karyopherin *KPNA2* showed a 3.8-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *KPNA2* was confirmed by RT–PCR and Western blot analyses. The active transport of proteins into the nucleus requires an array of proteins, including karyopherin. KPNA2 is one of six forms of karyopherin alpha proteins. Recently, it was shown that KPNA2 is able to interact with the p65 subunit of nuclear factor-κB (NF-κB) (Cunningham *et al.*, 2003). NF-κB activation requires removal and degradation of its inhibitor κB, an event that occurs after phosphorylation of inhibitor κB by a complex of inhibitor κB kinases. These events allow NF-κB to translocate into the nucleus, to bind DNA, and regulate gene transcription. NF-κB nuclear translocation plays an important role in preventing apoptotic cell death in some cancers. Recently, it was shown that inhibition of NF-κB enhances apoptosis in human lung adenocarcinoma cells *in vitro* (Milligan and Nopajaroonsri, 2001). Therefore, it is possible to hypothesize that downregulation of *KPNA2* could evade, in part, NF-κB nuclear translocation and subsequently, its antiapoptotic effect.

MAGE genes were initially identified because they encode tumor antigens that are recognized by cytolytic T lymphocytes derived from blood lymphocytes of cancer patients (Van den Eynde and van der Bruggen, 1997). The *MAGE* gene family is composed of 23 related genes divided into four clusters. The *MAGE-A* subfamily is very appealing for its potential as antitumor immunotherapic because MAGE-A proteins are strictly tumor specific. Seven *MAGE-A* genes (*MAGE-A1*, *-A2*, *-A3*, *-A4*, *-A6*, *-A10*, and *-A12*) have been found highly transcribed in a large proportion of tumors of various histological origins, such as metastatic melanomas, testicular germ cell tumors, lung cancer, sarcoma, mammary tumors, and colon carcinomas (Ohman Forslund and Nordqvist, 2001). Recently, *MAGE-A1*, *3*, *6*, *12*, and *4b* subfamily members have also been proposed as candidate biomarkers in lung carcinoma (Sugita *et al.*, 2002). Our microarray analysis revealed that *MAGE-A 3/6* genes are downregulated with a ratio of 2.5 and these data were confirmed by Western blot analysis, suggesting an interesting link between *MAGE-A 3/6* genes, *RB2/p130*, and lung cancer.

MKK3 showed a 15.8-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *MKK3* was confirmed by RT–PCR and Western blot analyses. MKK3 specifically activates p38-MAPK that regulates inflammation, apoptosis, and development (Schaeffer and Weber, 1999). Very recently, it has been shown that p38-MAPK is activated in NSCLC, suggesting an additional role of this kinase in malignant cell growth or transformation (Greenberg *et al.*, 2002). Additionally, *MKK3* has also been found to be homozygously deleted in a lung tumor cell line. All these data suggest that there is a close relation between this gene and lung carcinogenesis (Teng *et al.*, 2001).

NIK showed a 6.6-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *NIK* was confirmed by RT–PCR and Western blot analyses. *NIK* is a protein kinase able to upregulate *NF-κB* (Malinin *et al.*, 1997) that is highly expressed in NSCLC (Mukhopadhyay *et al.*, 1995), playing an important role in anchorage-independent and metastatic growth of lung carcinoma cells (Jiang *et al.*, 2001).

The proto-oncogene Pim-1 (*PIM-1*) showed a 2.2-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *PIM-1* was confirmed by RT–PCR analysis. *PIM-1* is a serine/threonine kinase that when overexpressed is involved in lymphomagenesis. *PIM-1* also associates with protein complexes necessary for mitosis and maps to an area on chromosome 6, which is

close to a region that is amplified in NSCLC (Bhattacharya *et al.*, 2002).

Proliferating cell nuclear antigen (*PCNA*) showed a 16.9-fold reduction of expression with respect to the control 48 h after pRb2.p130 transduction by microarray analysis. Downregulation of *PCNA* was validated only by RT–PCR. PCNA is a nuclear protein acting as a cofactor for DNA polymerase delta (Mathews *et al.*, 1984). PCNA has been used as a proliferation and prognostic marker in a variety of malignancies, including lung tumors (Kawai *et al.*, 1994) and it is an important prognostic factor of clinical outcome in patients with NSCLC (Volm and Koomagi, 2000).

Unfortunately, PCNA has not lived up to its early promise as a cellular proliferation marker. The relatively long half-life of the protein extends its expression beyond the S phase into the M phase and beyond again into the G0 phase of cells in rapidly proliferating tumors. This could in part explain why we did not find protein downregulation when compared to its transcriptional modulation following RB2/p130-enhanced expression. In fact, recently Markey *et al.* (2002) found that in a doxocycline-inducible RB1 system, PCNA does not change at the protein level after 48 h from induced RB1 expression, suggesting that the failure to change could reflect a relatively long PCNA half-life.

PLK1 showed a sevenfold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *PLK1* was confirmed by RT–PCR and Western blot analyses. *PLK1* is a member of PLKs serine–threonine kinases that are highly conserved during evolution. More evidence supports the concept that Plks regulate different cell-cycle stages throughout mitosis, including its initiation by activating Cdc2 through Cdc25 and direct phosphorylation of cyclin B1 targeting Cdc2/cyclin B1 to the nucleus. Moreover, Plks are key regulators of cytokinesis (Glover *et al.*, 1998; Nigg, 1998). *PLK1* is involved in centrosome maturation, DNA damage checkpoint adaptation, bipolar spindle formation, and activation of Cdc16, Cdc27 as components of the anaphase-promoting complex for mitotic exit. *PLK1* is overexpressed in various human tumors, for example, NSCLC, squamous cell carcinomas of the head and neck, melanomas, endometrial, and ovarian carcinomas (Yuan *et al.*, 1997, 2002). It has also been recently shown that the constitutive expression of *PLK1* may contribute to cancer progression (Smith *et al.*, 1997). *PLK1* overexpression is also a negative prognostic factor in patients suffering from NSCLC (Wolf *et al.*, 1997). All these data, taken together, suggest for the first time a link between a member of the *PLK* family, *RB2/p130*, and lung cancer.

RAF-1 showed a 2.1-fold reduction of expression with respect to the control 48 h after pRb2/p130 transduction by microarray analysis. Downregulation of *RAF-1* was validated by RT–PCR and Western blot analyses. The serine/threonine kinase Raf-1 is the first downstream effector of Ras to be identified. Activation of this kinase is necessary and sufficient for the activation of a kinase cascade consisting of *MEK1,2* and *ERK 1,2* (Marshall, 1994). Activation of the pathway *RAF1/MEK/ERK* plays an important role in gene expression control during cell cycle, apoptosis, cell differentiation, and migration and is frequently associated with transformation of primary cells and tumor progression. In fact, recent studies suggest a possible role of Raf-1 in lung tumorigenesis (Yano *et al.*, 1999; Ramakrishna *et al.*, 2002).

In a typical cell, it has been estimated that most of the expressed genes can be expressed in abundance class at (or below) the detection threshold of microarray technology. This includes many genes that do not need to be expressed at extremely high levels in order to be biologically active, such as growth factors and transcriptional regulators (Fambrough *et al.*, 1999). Therefore, a more sensitive method such as semiquantitative RT–PCR analysis could be an acceptable strategy, not only to validate microarray data but also to identify significantly induced genes whose transcripts belong to lower abundance classes.

Our microarray analysis indicates that adenovirus-mediated enhanced expression of the tumor suppressor gene *RB2/p130* in the H23 human lung cancer cell line downregulated a variety of genes involved in many cellular processes including cell division, cell signaling/cell communication, cell structure/motility, gene expression, and metabolism. Adenovirus-mediated expression of pRb2/p130 upregulated a cluster of 28 genes with an average ratio between 2.0 and 8.5 (data not shown), and some of them have been found to be previously linked to lung cancer. Further studies are needed to investigate the significance of these genes' regulation by pRb2/p130 better.

This study identifies a cluster of genes that are modulated by Rb2/p130 expression. Although part of these genes could not be the target of pRb2/p130 at its physiological level, most of them might mediate new potential therapeutical effects of RB2/p130 in lung cancer. Therefore, we feel that these studies, by bringing about a better understanding of ample spectra of proteins' expression, are essential because they could identify new cancer biomarkers and could facilitate matching the appropriate therapies to lung cancer, thereby maximizing therapeutic efficacy and minimizing toxicity.

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All publications mentioned in this specification are indicative of the level of those skilled in the art to which this invention pertains. The contents of all the publications are incorporated herein by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

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